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INVESTIGATIONS OF THE CAPACITY OF SYNTHESIZING 3β-STEROLS IN MOLLUSCA—XIII. BIOSYNTHESIS AND COMPOSITION OF STEROLS IN SOME BIVALVES (ANISOMYARIA)

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Abstract—1. The incorporation of sodium acetate-1- or -2-^{14}C into some lipid classes of the anisomyarian bivalves *Mytilus edulis*, *Atrina fragilis* and *Ostrea edulis* was investigated.

2. It was demonstrated that the acetate was utilized for the biosynthesis of both saponifiable and non-saponifiable lipids.

3. Squalene could not be detected by means of gas–liquid chromatography.

4. Sterols were not synthesized by the animals during the experiments.

5. The sterol composition of the animals was determined. Sterols with 27 carbon atoms were the main ones (45–50 per cent), followed by those with 28 carbon atoms (35–37 per cent). Cholesterol and brassicasterol were quantitatively the most important sterols.

INTRODUCTION

MANY investigators have studied the sterols of Bivalvia. The reasons for this interest are quite obvious. First, the animals possess economic value, and second, at the time their sterols were considered to be a rich source of provitamin D and actually a great deal of the studies on bivalve sterols was initiated with the aim to indicate new sources of these provitamins. Results have been summarized in several reviews (Toyama, 1958; Bergman, 1962; Austin, 1970; Idler & Wiseman, 1972; Voogt, 1972a). Unfortunately, most of these data are out of date and of little use because they were obtained before powerful separation and suitable identification techniques were available. Recent data have been given by Voogt (1972a). In the past few years interest in the sterols of bivalves has been increasing again (Idler & Wiseman, 1971a). This has led to the discovery of several new sterols (Idler *et al.*, 1970, 1971; Idler & Wiseman, 1971b; Teshima *et al.*, 1972).

Whereas our knowledge of the sterol composition in Bivalvia increased very fast, our knowledge of sterol metabolism is still very fragmentary and the results are difficult to interpret because they are contradictory.

Fagerlund & Idler (1960) reported that radioactivity was present in digitonin-precipitable material from *Mytilus californianus* and *Saxidomus giganteus* after the injection of sodium acetate-2-^{14}C. Most activity was present in Δ^5-monounsaturated sterols, with only traces in 24-methylenecholesterol (Fagerlund & Idler, 1961b). They also found that *Saxidomus giganteus* was able to synthesize 24-methylenecholesterol from cholesterol (Fagerlund & Idler, 1961b) and to introduce Δ^{22} and Δ^{25} double bonds (Fagerlund & Idler, 1961a). From their study on the seasonal variations of the sterols of *Placopecten magellanicus*, Idler *et al.* (1964) concluded that in this animal metabolism and synthesis of cholesterol and 24-methylenecholesterol were interrelated. Tamura *et al.* (1964) obtained indications that *Crassostrea* is able to synthesize cholesterol and to convert this into 24-methylenecholesterol. Thus all the results obtained by Idler and coworkers suggest that in bivalves there is an active sterol metabolism and that these animals are able to synthesize sterol(s). On the other hand, Salaque *et al.* (1966) reported that after incubation of *Ostrea gryphea* in either L-methionine-5-^{14}C or DL-mevalonic acid-2-^{14}C, no radioactivity was present in the sterols. Walton & Pennock (1972) concluded from their experiments that *Cardium edule* and *Mytilus edulis* were not able to synthesize sterols from DL-mevalonate-2-^{14}C.

In the scope of the long-term project of our laboratory on the biosynthesis of sterols in invertebrates, we started investigation of bivalves in 1966. Whereas, in the data mentioned above, there is no discrimination between reports on Anisomyaria or Eulamellibranchia (most reports are concerned with Anisomyaria), we decided to study both orders separately. This paper deals with some Anisomyaria, viz. *Ostrea edulis*, *Mytilus edulis* and *Atrina fragilis*.

It contains the results of the experiments on their capacity of synthesizing sterols and further the sterol compositions determined by means of gas liquid chromatography.

MATERIALS AND METHODS

Specimens of *Mytilus edulis* were collected on old oysterbanks at Kattendijke (Zeeland, The Netherlands), those of *Ostrea edulis* were obtained from the Netherlands Institute of Sea Research at Texel, while those of *Atrina fragilis* were collected by fishermen of the Laboratory for Marine Biology Laboratoire Arago at Banyuls-sur-Mer, France.

The animals were each injected with an aqueous solution of either sodium acetate-1-^{14}C or sodium acetate-2-^{14}C. Injections were given directly into the hepatopancreas (in the case of *Atrina*) or into the foot (in all other cases). In the experiment indicated *Ostrea* III animals were injected on the first, the third and the fifth day. The animals were incubated in running sea water for varying times and then killed. They were stored at −20°C or fixed in ethanol until they were used. Data about the animals and the radioactive precursor administered are given in Table 1.

Lipids were extracted from the animals using the procedure of Bligh & Dyer (1959) with the lipids being saponified in a solution of 1·5 N KOH in 80% methanol under the usual conditions. In the experiment called *Ostrea* I the animals were saponified *in toto* in this solution. Unsaponifiable and saponifiable lipids were isolated from the saponification mixture in the usual way. The unsaponifiable lipids were separated into a hydrocarbon fraction, a crude sterol fraction and a remaining fraction by means of column chromatography on alumina (Voogt, 1971a, b).

The hydrocarbon fraction was analysed on a Becker gas chromatograph, Model 1452 or 2300. The glass columns (180 × 0·38 cm i.d.) were filled with Chromosorb W (80–100 mesh, acid washed and silanized) coated with 1% SE-52. In addition, the hydrocarbon fraction was hydrogenated in iso-octane as described previously (Voogt, 1971b) and then chromatographed. Some carrier squalene was added to the hydrocarbon fraction and the squalene hexahydrochloride was prepared (Loud & Bucher, 1968).

Sterols were isolated from the crude sterol fraction via their digitonides and purified by recrystallizing them from ethanol. Sterols of *Ostrea* III were also purified by means of thin-layer chromatography, sterols being chromatographed thrice on Silicagel G using for development consecutively toluene–diethylether–ethanol–acetic acid (50 : 40 : 2 : 0·2, by volume) di-isopropylether–acetic acid (98 : 2, v/v) and toluene–ethylacetate (4 : 1, v/v).

Gas chromatography and identification of the 3β-sterols were performed as given elsewhere (Voogt, 1972b).

Radioactivity was determined in a Packard Tri-Carb-Liquid Scintillation Spectrometer, Model 3315. The scintillation medium consisted of toluene containing

Table 1. Data about some bivalves examined for their capacity of synthesizing sterols

	Mytilus edulis		*Atrina fragilis*	*Ostrea edulis*		
	I	II		I	II	III
Number of animals	61	100	2	6	8	2
Date of collection	26 April 1967	14 June 1968	21 June 1967	2 Feb. 1966	16 March 1966	25 Nov. 1966
Radioactive precursor (specific radioactivity)	Sodium acetate-1-^{14}C (21·6 mCi/mM)	Sodium acetate-2-^{14}C (2 mCi/mM)	Sodium acetate-1-^{14}C (20 mCi/mM)	Sodium acetate-1-^{14}C (20 mCi/mM)	Sodium acetate-1-^{14}C (20 mCi/mM)	Sodium acetate-1-^{14}C (20 mCi/mM)
Dose administered (μCi)	3·5	1	25	25	25	3 × 25*
Incubation time (hr)	120	72	56	6·5	4·5	168

* Explanation in text.

Table 2. Quantities and relative weights of the lipid fractions isolated from some bivalves

	Mytilus edulis		*Atrina fragilis*	*Ostrea edulis*		
	I	II		I	II	III
Fresh weight* (mg)	354,000	392,000	92,000	295,700	242,000	210,000
Total lipids (mg);	5091·7	6127·7	533·2	2799·3†	3321·5	8191·0
% fresh wt.	1·44	1·56	0·58	0·95	1·37	3·90
Unsaponifiable lipids (mg);	515·5	798·0	99·7	964·7	477·1	448·0
% fresh wt.	0·15	0·20	0·11	0·33	0·20	0·21
Hydrocarbon fraction (mg)	30·0	25·3	11·2		18·4	19·3
Crude sterol fraction (mg)	389·9	522·0	51·7	307·2	231·2	180·0
3β-Sterols (mg);	314·2	448·6	16·6	223·9	179·1	153·6
% fresh wt.	0·09	0·11	0·02	0·08	0·07	0·07

* Fresh weight, determined without the shells.

† Determined by summation of unsaponifiable and saponifiable lipids.

Table 3. Radioactivity in the lipid fractions isolated from some bivalves after the injection of sodium acetate-1- or -2-^{14}C expressed in dis min^{-1} mg^{-1}

	Mytilus edulis		*Atrina fragilis*	*Ostrea edulis*		
	I	II		I	II	III
Total dosage administered (dis/min)	$4{\cdot}74\times10^8$	$2{\cdot}22\times10^8$	$1{\cdot}11\times10^8$	$3{\cdot}33\times10^8$	$4{\cdot}44\times10^8$	$3{\cdot}33\times10^8$
Total lipids		418·5	2581·1		3450·6	1195·0
(incorporation as percentage of the dosage administered)		1·16	1·24		2·58	2·94
Saponifiable lipids	4396	325·0	3570·2	819·0	4397·3	1430·9
Unsaponifiable lipids	439·0	109·9	576·7		2131·3	840·8
Hydrocarbon fraction	40·8		159·2	392·0	695·0	46·5
Crude sterol fraction	106·3	51·4	99·9	26·3	886·7	258·7
3β-sterols	7·6	8·4	8·9	1·1	374·0	264·8
3β-sterols after three recrystallizations	7·2	6·2	4·1	1·4	212·7	308·0
3β-sterols after four recrystallizations	6·8	5·8	0		289·0	348·3
3β-sterols after five recrystallizations					325·2	

4 g 2,5-diphenyloxazole (PPO) and 50 mg 1,4-di-2-(-5-phenyloxazolyl)-benzene (POPOP) per litre (Hayes *et al.*, 1956).

RESULTS AND DISCUSSION

The quantities of the isolated lipid fractions were determined and are given in Table 2. This table shows that generally the lipid contents in bivalves are low, ranging from 1·0 to about 1·5 per cent of the fresh weight. Very different values were found for *Atrina* and *Ostrea* III. The relative amounts of unsaponifiable lipids and 3β-sterols obtained for *Atrina* (Table 2) show that this animal is indeed poor in lipid, whereas the corresponding values obtained for *Ostrea* III are in good agreement with those of the other animals and suggest that the high lipid content in this case is due to the presence of saponifiable lipids, for example glycerides from the depot fat of the animals.

The specific radioactivity of each lipid fraction was determined and the results are shown in Table 3.

This table shows that incorporation of radioactivity into lipids is low. Even after the repeated injection of acetate in *Ostrea* III—finally to a dose of 75 μCi per animal and after an incubation time of 168 hr—incorporation of radioactivity into the lipids was no higher than 2·94 per cent. In all cases both saponifiable and non-saponifiable lipids were radioactive, allowing the conclusion that the animals are able to synthesize these lipids from acetate.

The hydrocarbon fractions were analysed on the stationary phase SE-52. In the chromatograms a series of peaks—probably representing a series of homologous hydrocarbons—was present, but squalene could not be detected, neither could squalane in chromatograms of the hydrogenated hydrocarbons. Some carrier squalene was added to the hydrocarbons remaining and squalene hexahydrochloride was prepared. This product appeared to be non-radioactive. This means that squalene was not synthesized under the experimental conditions.

Table 3 shows that in contrary to the sterols of *Mytilus*, *Atrina* and *Ostrea* I, those of *Ostrea* II and III are rather radioactive. The specific radioactivity of these sterols increased on recrystallization and had not yet reached a constant value even after four or five recrystallizations. At the same time, the melting point of the sterols decreased. The following melting points were observed for *Ostrea* II: after one recrystallization 136·3°C, after two 129.3°C, after three 128·7°C and after five 121·8°C. This indicated that a radioactive component, which was not necessary a sterol, was enriched. For this reason the sterols of *Ostrea* III were also purified by means of thin-layer chromatography. The initial specific radioactivity amounted to 289 diss/min per mg. After chromatography in toluene–diethylether–ethanol–acetic acid specific radioactivity had decreased to 107 diss/min per mg. After an additional purification with toluene–ethylacetate the specific radioactivity amounted to 75 diss/min per mg and after a third purification in disopropylether–acetic acid this value was 63 diss/min per mg. This also confirmed that purification of the sterols of *Ostrea* was difficult. That is why the sterols were subjected to preparative gas–liquid chromatography (PGLC). Now radioactivity was eluated from the column before any of the sterols appeared. Further sterols were hydrogenated and their acetates purified via PGLC. This yielded the same results, viz. radioactivity was not concurrent with the sterols. From this it can be concluded that in *Ostrea* III, too, sterols are not radioactive. Sterols of *Ostrea* II were not available to subject them to PGLC, but it is

reasonable to suppose that they would have shown the same picture.

Summarizing we can say that in our experiments no radioactivity was incorporated into sterols either from sodium acetate-1-^{14}C or from sodium acetate-2-^{14}C and that in *Ostrea*, where initially sterols were radioactive, finally radioactivity turned out to be not concomitant with the sterols. Thus our results are not consistent with those of Idler and coworkers, who concluded that biosynthesis of sterols occurred in *Mytilus californianus* and *Crassostrea virginica* but are in full agreement with those of Salaque *et al.* (1966). Yet we are not allowed to conclude that anisomyarian bivalves are not capable of synthesizing sterols because sterol biosynthesis may proceed at an extremely low rate in these animals. However, experiments of Idler and his coworkers lasted for a much longer time and even then mostly only indications for sterol biosynthesis were obtained.

A small amount of the sterols of each species was hydrogenated and after acetylation analysed on the stationary phase SE-30. In all chromatograms five peaks were present, representing sterols with a carbon content ranging from twenty-six to thirty carbon atoms. The proportional compositions of the sterols were calculated and are given in Table 4.

Table 4. Proportional composition, according to carbon content, of the hydrogenated steryl acetates of some bivalves

	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}
Mytilus edulis	9·0	45·0	37·3	8·5	0·2
Atrina fragilis	3·7	50·2	35·7	9·7	0·8
Ostrea edulis	7·5	49·6	37·5	5·2	0·2

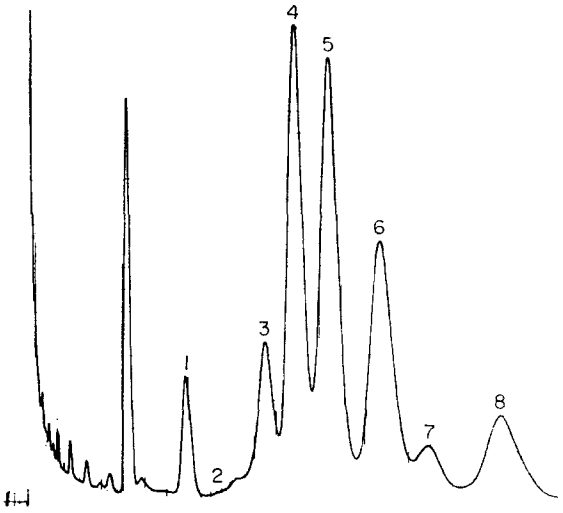

Fig. 1. Chromatogram of TMS derivates of the sterols of *Mytilus edulis* after GLC separation on the stationary phase SE-30. The percentage composition and the steroid number of each sterol are given. The steroid numbers of the suitable sterols are given in parentheses. 1. 5·9% C_{26} sterol 28·54; 2. 12·7% 22-dehydrocholesterol 29·85 (29·80); 3. 35·9% cholesterol 30·21 (30·18); 4. 31·5% brassicasterol 30·58 (30·58) or desmosterol (30·50); 5. 9·9% campesterol 31·11 (31.19) or 24-methylenecholesterol (31·07); 6. 1·5% stigmasterol 31·52 (31·50); 7. 2·3% β-sitosterol 32·06 (32·04) or 28-isofucosterol (32.02).

The sterols were analysed on the stationary phases SE-30 and NPGS as their trimethylsilylethers. Chromatograms of the three species resembled each other very closely. For this reason only representative chromatograms of *Mytilus* are depicted (Figs. 1 and 2). The proportional com-

Table 5. Proportional composition of the sterols of some bivalves

Sterols	*Mytilus edulis*	*Atrina fragilis*	*Ostrea edulis*
C_{26} sterols	5·0*	3·6*	7·5*
22-*cis*-Cholesta-5,22-dien-3β-ol	1·0	7·8	3·6
22-*trans*-Cholesta-5,22-dien-3β-ol	9·0	11·2	11·6
Cholest-5-en-3β-ol	34·0	31·1	33·2
Cholesta-5,24-dien-3β-ol	10·5	0·9	?
24-Methyl-cholesta-5,22-dien-3β-ol	21·5	23·2	24·7
24-Methyl-cholest-5-en-3β-ol	3·0	1·8	2·6
24-Methylenecholest-5-en-3β-ol	9·0	7·3	9·5
24-Ethyl-cholesta-5,22-dien-3β-ol	3·0	2·7	1·5
24-Ethyl-cholest-5-en-3β-ol	3·5	5·1	3·4
24-Ethylidenecholest-5-en-3β-ol	0·3	—	1·7
Unidentified	0·3	5·4	0·4

* Two components.

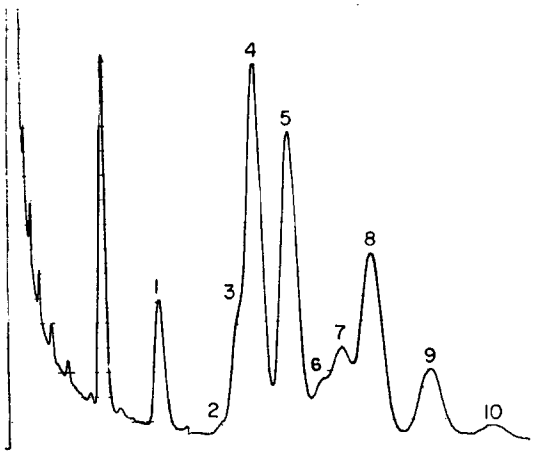

Fig. 2. Chromatogram of TMS derivatives of the sterols of *Mytilus edulis* after GLC separation on the stationary phase NPGS. The percentage composition and the steroid number of each sterol are given. The steroid numbers of the suitable sterols are given in parentheses. 1. 4·8% C_{26} sterol 28·60; 2. 1·0% 22-*cis*-dehydrocholesterol 29·87 (29·92); 3. 8·7% 22-*trans*-dehydrocholesterol 30·10 (30·09); 4. 33·0% cholesterol 30·34 (30·36); 5. 21·5% brassicasterol 30·77 (30·83); 6. 13·5% desmosterol 31·28 (31·15) or campesterol (31·43); 7. 12·0% 24-methylenecholesterol 31·72 (31·67) or stigmasterol (31·72); 8. 4·0% β-sitosterol 32·25 (32·29); 9. 0·3% 28-isofucosterol 32·78 (32·79).

position of the sterols was calculated and is summarized in Table 5.

Tables 4 and 5 show the strong resemblance in the sterol composition of these bivalves, desmosterol being the sole component which is present in widely different amounts. The composition of *Mytilus* is in good agreement with that of *Mytilus edulis* given by Idler & Wiseman (1971), although the latter authors do mention some lesser components with 28 and 29 carbon atoms. The C_{26} sterols given in Table 5 probably are identical with 22-*cis*- and 22-*trans*-24-norcholesta-5,22-dien-3β-ol. Possibly trace amounts of desmosterol are present in *Ostrea edulis*. In each of the three species (mostly) a minor component was present that could not be identified. It is striking that in bivalves C_{27} sterols do not hold the predominant position that they have in most other molluscs. Especially has cholesterol, which in most other molluscs makes up for over 50 per cent of the total sterols, become less predominant. At the same time brassicasterol has become more important. This suggests that brassicasterol might replace cholesterol in some of it functions. In general, we can say that in comparison to other molluscs there is a strong shift from mono-unsaturated sterols towards di-unsaturated ones. It would be interesting to know whether this phenomenon is related to the high content of poly-unsaturated fatty acids in bivalves (Voogt, 1972a).

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Key Word Index—Sterol biosynthesis in molluscs; sterols of bivalves; *Mytilus edulis*; *Atrina fragilis*; *Ostrea edulis*.